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54 Human Interferon beta2A and Interferon-beta2B, vectors containing genes coding for said Interferons, cell lines producing same and use of said Interferons as pharmaceuticals.

⑦ Human interferon-beta_{2A} and interferon-beta_{2B} are produced in purified form by recombinant DNA techniques. Two separate human genes have been identified which code for the production of IFN-β_{2A} and IFN-β_{2B}, respectively. The sequence of IFN-β_{2A} cDNA is established. These genes and cDNA have been cloned into mammalian cells with an SV40 early promoter sequence and such genomic clones are capable of producing IFN-β_{2A} and IFN-β_{2B}. The antiviral activity of such recombinant IFN-β_{2A} and IFN-β_{2B} is demonstrated as well as other biological activity identifying them as human interferons. It has been shown that IFN-β₂ secretion is induced in human cells by growth-stimulatory cytokines.

STRUCTURE OF HUMAN IFN β 2 GENE IFA-2

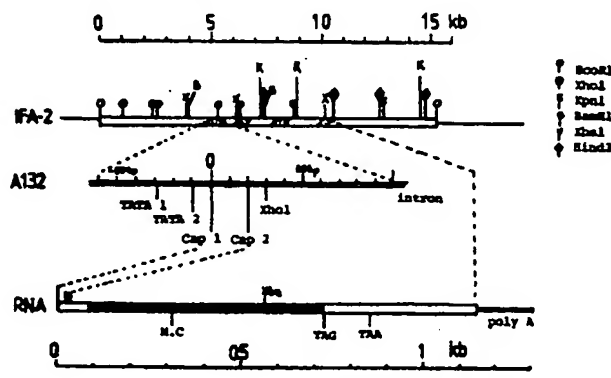


FIG.2

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HUMAN INTERFERON- β_{2A} AND INTERFERON- β_{2B} ; VECTORS CONTAINING GENES CODING FOR SAID INTERFERONS; CELL LINES PRODUCING SAME AND USE OF SAID INTERFERONS AS PHARMACEUTICALS

Interferon is an important antiviral and antitumor protein produced by the human body. Because of its species specificity, clinical use of interferon requires human interferon. Based on their neutralization by antibodies three types of interferon were defined which include interferon-alpha, produced mainly by leucocytes, interferon-beta, produced mainly by fibroblasts, and interferon-gamma, produced mainly by T-lymphocytes.

The major species of interferon produced by human fibroblasts in response to ds RNA is the 20 Kd glycoprotein IFN- β_1 , encoded by a 0.9 kb RNA which is transcribed from an intron-less gene on chromosome 9. However, additional mRNAs which yield IFN activity when micro-injected into frog oocytes have been observed in human fibroblasts induced by poly (rI)(rC) and by a sequential cycloheximide (CHX)-actinomycin D treatment. While cloning the IFN- β_1 cDNA, cDNA clones of another co-induced 1.3 kb RNA have been isolated which in reticulocyte lysates code for a 23-26 Kd polypeptide and in oocytes produce human IFN antiviral activity (J. Weissenbach et al. 1980 Proc. Natl. Acad. Sci. USA 77:7152-7156; British patent 2 063 882).

Since this activity was inhibited by anti-IFN- β_1 antibodies, it was designated IFN- β_2 . The protein product of IFN- β_2 RNA is immunologically distinct from IFN- β_1 , although the biological activities of the two proteins are cross-neutralized by the same antibodies. The IFN- β_2 1.3 kb RNA clearly originates from another gene than IFN- β_1 , since IFN- β_2 RNA is formed in mouse-human hybrids lacking chromosome 9 (U. Nir (1984) Ph.D. Thesis, Weizmann Institute of Science, Rehovot, Israel).

In the German patent application P 30 43 981 a process is disclosed for the preparation of interferon- β_2 in purified form. Said interferon- β_2 is produced by isolating DNA containing the nucleotide sequence coding for interferon- β_2 in human cells after cultivating those cells which are able to produce interferon- β_2 when exposed to an inducer of interferon. mRNA from said induced cells is extracted and purified. Afterwards, the mRNA is transcribed into DNA and the DNA is cloned in a suitable vector.

It could be demonstrated that there is a second gene encoding IFN- β_2 , which, when isolated and transfected into hamster or mouse cells produces human-specific IFN activity after induction by ds RNA and cycloheximide. The IFN produced has the properties ascribed to IFN- β_2 but does not have identical amino acid sequences, the proteins produced by the two genes are denominated IFN- β_{2A} and IFN- β_{2B} respectively.

Accordingly, it is an object of the present invention to isolate biologically active human IFN- β_{2A} and IFN- β_{2B} .

It is another object of the present invention to enable the production of biologically active human IFN- β_{2A} and IFN- β_{2B} .

It is yet another object of the present invention to prepare cDNA which codes for human IFN- β_2 , and, more particularly, cDNA which codes for human IFN- β_{2A} and cDNA which codes for human IFN- β_{2B} .

It is a further object of the present invention to identify genes hybridizing to IFN- β_2 cDNA.

It is still another object of the present invention to identify genes hybridizing to IFN- β_{2A} cDNA and to IFN- β_{2B} cDNA.

It is yet a further object of the present invention to isolate genomic clones containing an IFN- β_2 gene, and, more particularly, a genomic clone containing an IFN- β_{2A} gene and a genomic clone containing an IFN- β_{2B} gene.

It is another object of the present invention to provide a recombinant vector containing an IFN- β_{2A} and/or IFN- β_{2B} gene and a promotor sequence capable of exerting transcription control over the interferon gene.

It is yet another object of the present invention to provide cells which produce biologically active human IFN- β_{2A} and/or IFN- β_{2B} .

It is another object of the present invention to provide such cells which produce substantial quantities of such IFN- β_{2A} and/or IFN- β_{2B} .

It is still another object of the present invention to provide a process for the production of biologically active human IFN- β_{2A} and/or IFN- β_{2B} by recombinant DNA technology.

It is a further object of the present invention to provide genes which, when fused to a strong viral transcriptional promoter and transfected into hamster or mouse cells, produce substantial quantities of biologically active human IFN- β_{2A} and/or IFN- β_{2B} .

It is still another object of the present invention to use IFN- β_{2A} and/or IFN- β_{2B} in pharmaceuticals which are useful wherever it is desired to influence cell growth and differentiation, especially during terminal differentiation of cancer cells.

A further object of the invention is the use of IFN- β_{2A} and/or IFN- β_{2B} to inhibit fibroblast proliferation, to prevent sclerosis after infection.

In accordance with the present invention, the existence of biologically active human interferon-beta₂ - (IFN- β_2) molecules has been confirmed and these molecules are produced by recombinant DNA technology. IFN- β_2 is a glycoprotein secreted by human fibroblasts which are induced by double-stranded (ds) RNA or viruses to produce interferon. IFN- β_2 amounts to about 5% of the total IFN activity produced by fibroblasts. The amino acid sequence of IFN- β_2 , which was determined by cDNA sequencing, indicates only about 20% overall homology with that of the other human IFNs produced by such human cells (IFN- β , and IFN- α s).

A human cDNA or gene encoding IFN- β_{2A} may be cloned in accordance with the present invention and fused to a strong viral transcriptional promoter. Hamster cells transformed by the fused cDNA or gene produce constitutively human-specific interferon activity which inhibits viral replication and cytopathic effect and induces the proteins typical of the biological response of human cells to interferons. A second gene encoding IFN- β_2 is also disclosed which, when isolated and transfected into hamster or mouse cells, produces human-specific IFN activity after induction by ds RNA and cycloheximide. As the IFN produced by both genes have the properties ascribed to IFN- β_2 , but do not have identical amino acid sequences, the proteins produced by the two genes are denominated IFN- β_{2A} and IFN- β_{2B} respectively.

Description of the drawings

Figure 1 shows the restriction map, sequencing strategy and nucleotide sequence of IFN- β_{2A} cDNA determined from clone AE20, formed by fusing E474 cDNA and A341 cDNA clones (Weissenbach et al. - (1980), supra) by their Xba-1 site. The 5' end sequence was completed from the genomic clone IFA-2 shown in Fig. 2. Numbering begins from the S-1 start of Fig. 3 and 4. The amino acid sequence of the IFN- β_{2A} protein is deduced.

Figure 2 shows the structure of genomic clone IFA-2 containing the IFN- β_{2A} gene. A132 is a subclone of an EcoRI segment of IFA-2 in pBR322. Shaded areas are gene regions hybridizing to cDNA clones A341 and E474 (Weissenbach et al. (1980), supra). The positions of the two RNA starts (cap 1 and 2) and the TATA boxes are shown. In the RNA the longest open reading frame (ORF) of 212 amino acids (Fig. 1) is shown in black. M.C. indicates a second internal ATG in the same frame. No other long ORF are found in the entire IFN- β_{2A} cDNA (Fig. 1).

Figure 3 shows the S1 nuclease analysis of 5' starts in IFN- β_2 RNA. The DNA probe, a fragment from subclone A132 of IFA-2 gene (Figure 2) labelled at BstNI site was hybridized to total RNA from diploid fibroblasts treated (from left to right) by: priming with IFN- β , 200 U/ml, 16 hrs; priming and poly (rI)(rC)(pIC) 50 μ g/ml, 3.5 hours; priming and cycloheximide (CHX) 50 μ g/ml, 3.5 hours; priming and pIC plus CHX 3.5 hours; priming and CHX for 6.5 hours; no priming and pIC 3.5 hours; same CHX 3.5 hours; same pIC plus CHX 3.5 hours. S-1 and S-2 are the signals generated by the 2 RNA starts of Figure 2.

Figure 4 shows the sequence of the promoter region of the IFA-2 GENE encoding IFN- β_{2A} . The sequence of part of subclone A132 (Figure 2) is shown compared to the IFN- β_2 cDNA. S1 and S2 are the 2 RNA starts determined in Figure 3. The initiator ATG is marked by X. The first intron starts at intr 1.

Figure 5 illustrates the fusion of IFN- β_{2A} cDNA to the SV40 early promoter. A plasmid pSVIFA2-II was first constructed by subcloning the 5' Xho-BamHI (Xh-B) segment of the IFA-2 gene (from 5.5 kb to 7.5 kb in Figure 2) fused with a Cla-Xho, synthetic adaptor of 26 bp (restoring the 5' cDNA sequence), into a Cla, and Bam HI-cut pBR plasmid. The 3' BamHI-HindIII (B-H) gene segment (from 7.5 kb to 10.5 kb in Figure 2), subcloned in a HindIII, BamHI-cut pBR plasmid, was excised using the Cla, site adjacent to HindIII in pBR322 and ligated to the above 5' segment to obtain the complete IFA-2 gene. A pSVE3 vector cut by Hind III as described in Y. Chernajovsky et al. (1984) DNA 3: 294-308, was reclosed with Cla, linkers - (forming pSVC1a) and the above IFA-2 gene introduced in the Cla, site of pSVC1a in the same orientation as the SV40 early promoter forming pSVIFA2-II. A plasmid pSVIFA2-I was similarly constructed but the Xho, site of IFA-2 was fused directly to the Cla, site of pBR before introduction in pSVE3. To obtain the pSVCIF β_2 plasmid containing the IFN- β_{2A} cDNA fused to the SV40 promoter, the above pSVIFA2-II DNA, cut by Xba, was partially cut by Xmn, to open the Xmn, site located 80 bp downstream from the Xho, site in the IFN- β_2 cDNA sequence (Figure 1), and not an Xmn, site located in the amp gene of the vector. To this construct, the Xmn-Xba, segment of IFN- β_2 cDNA clone AE20 (coordinates 92-566 in Fig. 1) was ligated,

restoring the complete IFN- β_{2A} cDNA sequence as shown in the Figure. EES is the early-early RNA start of SV40 T-ag gene; ATG is the IFN- β_2 initiation codon; pA are IFN- β_2 and SV40 polyadenylation sites, respectively. pSVCIF β_2 DNA was cotransfected with a pDHFR plasmid into CHO-K, DHFR⁻ cells as in Y. Chernajovsky et al. (1984), (supra) and clones were tested for human IFN antiviral activity in FS11 cells using Vesicular Stomatitis Virus (VSV). Gene amplification was obtained by selecting CHO clones for methotrexate (MTX)-resistance. Assay of the culture medium of CHO-SVCIF β_2 B132-5M cells, resistant to 250 nM MTX, is shown in the lower part of the Figure. After VSV cytopathic effect developed, cells in the microplate were stained by methylene blue. Serial two-fold dilutions of the medium are from left to right. By comparison to IFN standard (ST) and non-transfected CHO cells, a titer of 300 U/ml rIFN- β_2 is calculated.

Figure 6 shows the results of an assay of (2'-5') oligo A synthetase with poly (rI)(rC)-agarose bound NP40 extracts (M. Revel et al. (1981) *Meth. Enzymol.* 79:149-161) of human FS11 previously exposed for 16 hours to medium from a) Cos7 cells two days post-transfection with the pSVIFA2-II DNA (described in Figure 5) (leftmost 4 lanes), b) hamster CHO SI-15 cells stably transformed by the pSVIFA2-I DNA of Fig. 5 (next 4 lanes), c) CHO MEIF-5 cells expressing IFN- β_2 gene (Y. Chernajovsky et al. (1984), supra) (next 2 lanes); d) CHO cells with DHFR gene only (next lane); e) fresh medium (Non Ind.). The IFN antiviral activity measured per ml of the media added to FS11 cells is indicated. An electrophoresis of phosphatased, 32P- α -ATP labelled (2'-5') oligo A product is shown.

Figure 7 is a comparison of amino acid sequences between type I human IFNs. Amino acids that are conserved in IFN- β_2 and the various IFN- α s (shown are αA , αC , replacements in other α s excluding αE , and IFN- α class II), are marked by stars over the IFN- β_2 sequence. Amino acids conserved in IFN- β_2 are shown above its sequence by stars if conserved in all type I IFNs, or by squares if conserved in some IFNs. IFN- β_{2A} was aligned by comparing hydropathy plots (see text) and numbered from the presumed processing site.

Figure 8 shows restriction maps of the two different human IFN- β_2 genes which are expressed in rodent cells. The IFA-11 DNA (IFN- β_{2B} gene) was transfected into either CHO or L cells and expressed IFN- β activity after induction (Table 1).

Figure 9 shows an IFN- β_2 specific immunocompetition assay with R-antibodies to show that human fibroblasts FS11 cells secrete the IFN- β_2 protein in response to the two cytokines interleukin-1 (IL-1) and Tumor Necrosis Factor (TNF- α). Both cytokines induce the synthesis and secretion of the IFN- β_2 protein in two levels which have been estimated by the immunoassay to be 10 to 20 U/ml IFN- β_2 . Optimal IFN- β_2 induction was seen with the concentrations of rIL-1 α of 4 U/ml (0.13 ng/ml). The optimal concentration of TNF- α for IFN- β_2 induction (400 U/ml; 40 ng/ml) was higher than that required for cytolytic effect on sensitive cells (0.1 ng/ml) and even higher than that giving optimal growth stimulation of diploid fibroblasts - (2 ng/ml).

Detailed description of preferred embodiments

The existence of interferon-beta₂ was discovered in the course of attempts to isolate genetic material containing the nucleotide sequence coding for interferon in human fibroblast cells. In this process, fibroblast cells were induced for the production of inducible interferon mRNA with a suitable exogenous factor and then the mRNAs were extracted. At the same time the mRNAs from a non-induced culture of the same host cells were extracted. cDNA probes were synthesized from the mRNAs of both the induced culture and the non-induced control culture using the corresponding mRNAs as templates (induced cDNAs and non-induced cDNAs). Double-stranded cDNAs derived from the mRNA extracted from the induced culture were then synthesized and such cDNAs were inserted in appropriate vectors and transfected into suitable microorganisms. The microorganisms were cultivated under conditions suitable to cause selective development of microorganism colonies of the modified vectors (initial colonies). Duplicate colonies of the initial colonies were formed and the DNAs of both the initial and duplicate colonies were freed in situ. The DNAs of one of the two sets of duplicate colonies were hybridized with the cDNA probes synthesized from the mRNAs extracted from the induced culture and the other of the two sets of duplicate colonies were hybridized with the cDNA probes synthesized from the mRNAs extracted from the non-induced culture. Clones which selectively hybridized with the induced cDNA probes but not with the non-induced cDNA probes were selected and their DNAs recovered. These cloned DNAs were further tested to determine those DNAs capable of hybridizing with mRNA translatable in frog oocytes or reticulocyte-lysates into interferon, which DNAs are essentially those which code for interferon, or contain enough of the sequence coding for interferon to be used to obtain a complete interferon DNA.

During this investigation, it was discovered that two different mRNAs coding for interferon could be isolated from human fibroblast cells after being appropriately induced. Upon glycerol gradient centrifugation, the smallest mRNA sediments at 11S and yields by translation in a cell-free system a protein of molecular weight 20,000 which is selectively precipitated by antibodies prepared against one of the interferons that can be purified from these cells. This protein is human interferon-beta, of which the amino acid sequence was partially determined by E. Knight, et al. (1980 Science, 207, 525-526). The largest mRNA was found to sediment at 14S and yield a protein of molecular weight 23,000 which is precipitated by antibodies against a less purified preparation of fibroblast interferon. This protein is designated human interferon-beta₂. cDNA clones hybridizable to mRNA which translates to interferon-beta₂ were prepared in such a manner and an IFN-β₂ cDNA probe was prepared therefrom.

Two such IFN-β₂ clones A341 and E474 were found to represent overlapping sequencing and were fused to reconstitute the IFN-β₂ AE20 cDNA (Figure 1). The nucleotide sequence of this IFN-β₂ cDNA was determined and revealed an open reading frame of 212 amino acids predicting a protein of 23,500 daltons. Transcription-translation experiments confirmed that this cDNA encodes such a protein.

From a human gene library of human adult blood cell DNA cloned after partial EcoRI digestion in λ charon 4A (Y. Mory et al. (1981) Eur. J. Biochem. 120: 197-202), two genomic clones IFA-2 and IFA-11 hybridizing to such an IFN-β₂ cDNA probe were isolated. Clone IFA-2 contains a gene with at least 4 exons hybridizing to various segments of the cloned cDNA (Figure 1). An XhoI site present near the 5' of the AE20 cDNA allowed to map the genomic segment A132 (Figure 2) which contains the first IFA-2 exon ending 70 bp downstream from this XhoI site. By S1 nuclease analysis using DNA probes labelled at a BstNI site 40 bp after the XhoI site, it was determined that induced FS11 human fibroblasts contain IFN-β₂ RNA with two distinct 5' end S-1 and S-2 separated by 20 nucleotides (Figure 3). Each of these two starts is preceded in the IFN-2 gene by a potential TATA box at -30 (Figure 4). RNA ending at S-2 seems to be more abundant than the longer RNA under a variety of induction conditions (Figure 3). Both RNA 5' ends were also seen with cytoplasmic RNA instead of total cell RNA. Therefore, it is apparent that both RNAs are formed and active in human cells. Both IFN-β₂ RNAs are induced in human fibroblasts by poly (rI)(rC) and induction is stimulated by IFN priming as in the case of IFN-β₁ RNA in these cells (U. Nir et al. (1984) Nucl. Acids Res. 12: 6979-6993). A prolonged (6.5 h) treatment with CHX also induces IFN-β₂ RNA, but shorter - (3.5 h) CHX treatment is effective only with poly (rI)(rC) (Figure 3). The low induction of IFN-β₂ RNA by CHX without ds RNA distinguishes this gene from IFA-β₁.

DNA constructs were made to express the IFA-2 gene under the control of the SV40 early gene promoter. A 4.8 kb segment of IFA-2 DNA was fused through a synthetic oligonucleotide to pSVE3 DNA (Y. Chernajovsky et al. (1984), supra) so that the ATG codon is 150 bp downstream from the SV40' early RNA start (pSVIFA2-II, see legend, Figure 5). After transfection of pSVIFA2-II DNA into Cos7 monkey cells (Y. Gluzman (1981) Cell 23: 175-182), two RNAs of 1.35 and 2.2 kb were detected in Northern blots by IFN-β₂ cDNA; these two RNAs would correspond to transcripts ending at the IFN-β₂ and SV40 polyadenylation sites respectively (Figure 5). Medium from the Cos7 cells during transient expression of the pSVIFA2-II DNA was assayed for IFN activity by inhibition of the cytopathic effect of VSV on human FS11 and Wish cells as described in D. Novick et al. (1983) J. Gen. Virol. 64: 905-910. Antiviral activity could be clearly detected two days after transfection (Table 1). The specific antiviral activity of IFA-β₂ per unit protein has been estimated to be 30 to 100 times lower than that of IFN-β₁ and closer to that of IFN-α₁ (5-10 × 10⁶ units/mg). The IFN titers observed by transient expression of the IFA-2 gene were as expected, about 3% of those seen with the IFN-β₁ gene under the same SV40 promoter control (Table 1). Production of IFN-β₂ activity by pSVIFA2-II DNA cells was further demonstrated by induction of (2'-5') oligo A synthetase (M. Revel et al. (1981), supra) in FS11 cells exposed to the medium of the transfected Cos7 cells (Figure 6). The induction was dose-dependent and corresponded quantitatively to the antiviral activity.

The IFA-2 gene was also fused to the SV40 early promoter directly at the XhoI site near S-2 (pSVIFA2-I DNA, legend of Figure 5). Hamster CHO-K1 DHFR⁻ cells were cotransformed with pSVIFA2-I and pSVDHFR (Y. Chernajovsky et al. (1984), supra) DNAs and a stably transformed line SI-15 was isolated. This cell line produces constitutively 10-50U/ml of IFN antiviral activity measured on human FS11 and Wish cells, while CHO cells transformed only by pSVDHFR produce no human IFN (Table 1). By concentration of the medium from SI-15 cultures, rIFN-β₂ solutions titrating about 3000 U/ml could regularly be obtained. This rIFN-β₂ has the properties expected from a human IFN and induces the (2'-5') oligo A synthetase in FS11 cells even at low concentrations of 1 U/ml (Figure 6). The rIFN-β₂ constitutively produced by CHO SI-15 cells demonstrated human specific induction of the mRNAs for (2'-5') oligo A synthetase, C56 and HLA in the presence of cycloheximide, its antiviral activity was neutralized by anti-IFN-β₂ (but not anti-IFN-α or

anti-IFN- γ) polyclonal and monoclonal antibodies and it acted on mouse-human hybrids only if they contain human chromosome 21 which carries the gene for the type 1 IFN receptor. These further characterizations of the biological activity of rIFN- β_2 convincingly demonstrate that it acts directly as an IFN and is not an inducer of IFN- β_1 .

5 Improved yields of rIFN- β_2 were obtained with DNA constructs containing the IFN- β_{2A} cDNA sequence fused to the SV40 early promoter (pSVCIF β_2 shown in Figure 5), when this DNA cotransfected with pSVDHFR into CHO cells as above, was amplified by selecting cells resistant to increasing concentrations of methotrexate. Without amplification, the pSVCIF β_2 -transformed CHO cells (e.g. clone B-132) produced levels of IFN- β_2 activity similar to those produced by the SI-15 cells (Table 2). However, after selection for
10 resistance to methotrexate, higher yields were obtained with up to 800 U/ml (Table 2).

From such clones it was possible to immunoprecipitate the IFN- β_2 protein secreted into the culture medium and if the cells had been labelled by 35 S-methionine to analyse the IFN- β_2 protein by dodecyl sulphate polyacrylamide gel electrophoresis. The size of the secreted rIFN- β_2 was found to be 21,000 daltons. Indeed, experiments have shown that the 23-26 Kd primary translation product of IFN- β_2 RNA in
15 reticulocyte lysates is processed in vitro by dog pancreatic membranes into a shortened 21 Kd protein, and that induced human fibroblasts produce a 21 Kd protein which is immunologically similar to the IFN- β_2 RNA 26 Kd product, but distinct from IFN- β_1 . The N-terminus of the mature IFN- β_2 21 Kd protein has not been determined and two potential glycosylation sites are present in the IFN- β_2 sequence (Figure 1) making it difficult to calculate the size of the region removed by processing. However, the decrease in size indicates
20 that this processed region is longer than that in IFN- β_1 , the size of which increases by maturation (W. Degraeve et al. (1981) Gene 10: 11-15). A hydropathy plot of the IFN- β_2 26 Kd polypeptide has shown that there is a marked similarity between hydrophobic and hydrophilic regions of IFN- β_2 and of mature IFN- β_1 , when the two proteins are aligned by their C-termini. The same alignment also reveals conservation in the amino acid sequence of IFN- β_2 with those of the other type I human IFNs (Figure 7). Between all the known
25 IFN- α s and β , sequences, there are 38 amino acids which are conserved (marked by stars in Figure 7). When IFN- β_2 is aligned as above, 18 of these 38 amino acids common to all type I human IFNs can be seen to be conserved (Figure 7). There is an overall homology of about 20% between IFN- β_2 and the other type I IFNs.

Although the sequence homology between IFN- β_1 and β_2 is low, the cross-neutralization of the antiviral
30 activities of IFN- β_1 and IFN- β_2 by the same antibodies, including a monoclonal anti-IFN- β_1 (D. Novick et al. - (1983), supra), could result from some conserved structure in the active site of IFN- β . IFN- β_2 activity is not neutralized by anti-IFN- α or γ antibodies, justifying its designation as a β -type IFN. The genes encoding IFN- β_2 are not on chromosome 9 (U. Nir (1984), supra) and differ from the type I IFN genes in the chromosome 9 cluster by the presence of introns. The IFA-2 gene was determined to be on chromosome 7
35 (P.B. Sehgal et al. (1986) Proc.Natl.Acad.Sci. USA, in press). The amino acid sequence homology detected is compatible with the notion that the IFN- β_2 genes are related to the ancestral gene which gave rise to the type I IFN gene cluster.

The second genomic DNA clone IFA-11 hybridizing with the IFN- β_2 cDNA has a restriction map which differs from that of IFA-2 (Figure 8). Transcript mapping and sequencing showed that the 3' exon of IFA-2
40 and IFA-11 (XbaI-HindIII segment) are identical (99% sequence homology), while there was no cross-hybridization under stringent conditions between more 5' exons. The IFA-11 genomic clone (19 Kb) was transfected into L-TK⁻ cells together with a HSV-TK gene (F. Colbere-Garapin et al. (1981) J.Mol.Biol. 150: 1-14) and stable transformants were isolated. Table 1 shows that L cell clone LI-39 containing the human IFA-11 gene, produces human IFN activity when induced by poly (rI)(rC) and cycloheximide, while L-TK⁺
45 cells produced no such activity. A CHO clone 1C40 containing the IFA-11 gene also produced human IFN activity (Table 1). No activity was seen in non-induced LI-39 or 1C40 cells. The IFN activity obtained with IFA-11 transformants after induction was significantly higher than with the IFA-2 gene. The rIFN- β_{2B} produced by expression of the IFA-11 gene was neutralized with anti-IFN- β , similarly to the IFA-2 gene product. IFA-11 specific DNA probes hybridized to RNA from induced human fibroblasts similarly to IFN-2
50 DNA probes, indicating that both genes are active in human cells.

The function of the IFN- β_2 genes seems to result from their expression under conditions in which other IFN genes are not expressed. We find that fibroblasts exposed to Tumor Necrosis Factor (TNF) or to lymphokine IL-1 produce the IFN- β_2 protein. Others found that neutralization of IFN- β by antibodies results in a stimulation of cell proliferation (M. Kohase et al. (1986) Cell, in press; D. Resnitzky et al. (1986) Cell, in
55 press), suggesting that the IFN- β_2 acts to limit cell growth in response to growth factors. Supporting this conclusion is the fact that RNA hybridizing to IFN- β_2 cDNA was recently detected in peripheral blood mononuclear cells induced by mitogens such as PHA along with IFN- γ mRNA. It has also been reported that an IL-1 like lymphokine strongly induces RNA hybridizing to IFN- β_2 cDNA in diploid fibroblasts and

other human cell lines (J. Content et al. (1985) Eur.J.Biochem. 152: 253-257). The promoter region of the IFA-2 genes seems to contain two TATA boxes and two RNA starts (Figure 1 and 3). This IFN- β_2 promoter was shown to respond to either poly (rI)(rC) or to cycloheximide (Y. Chernajovsky et al. (1984), supra). In vivo, the two IFN- β_2 gene promoters may be activated by different inducers (ds RNA, protein synthesis inhibitors, IL-1 PHA). A variety of cells have been found to produce autocrine IFN- β in low amounts during growth arrest and differentiation (M. Revel (1983) Interferon 5 pp.205-239, Acad. Press, London). IFN- β_2 may belong to the same group of minor IFN species produced by cells to auto-regulate their growth, while the classical type I IFNs are probably more specifically involved in the response to viral infections.

The biological significance of IFN- β_2 lies most probably in the fact that it is induced under conditions where IFN- β_1 is not induced, as in metabolically stressed cells (A. Zilberstein et al., (1985) in The Interferon System, Serono Symposia Raven Press, pp.73-83). Most important is the observation that TNF induces IFN- β_2 in fibroblasts and that the proliferation of these cells which is stimulated by TNF is further enhanced if anti-IFN- β antibodies are added. A number of human cell lines have been shown to produce autocrine IFN- β species when undergoing differentiation, and these IFNs are responsible for the induction of HLA antigens on these cells (A. Yarden et al. (1984) Embo.J. 3: 969-973). In a mouse myeloleukemic cell line induced to differentiate by CSF-1, the growth arrest of the cells which characterized terminal differentiation, is abolished by anti-IFN- β antibodies. IL-1 stimulates growth of several cell types and the classical growth factor PDGF was found to induce IFN- β RNA in mouse cells (J.N. Zullo et al. (1985) Cell 43: 793-800). Although multiple IFN- β species may be involved (P.B. Sehgal, (1982) in Interferon 4, Academic Press, pp.1-22, and M. Revel (1983) in Interferon 5, Academic Press, pp. 205 -239), IFN- β_2 appears to be one of these autocrine autoregulators of cell growth produced in response to growth factors. This would be in line with the low specific activity of IFN- β_2 (estimated by comparison of immunoassay and antiviral activity to be 50-100 times lower than for IFN- β_1), since only very small antiviral activity is seen in cells producing the autocrine IFN- β species (M. Revel (1983) in Interferon 5, Academic Press, pp. 205-239). This could also explain why the hamster cells expressing IFN- β_2 cDNA produce less IFN activity than similar CHO cells harbouring the human IFN- β_1 gene (Y. Chernajovsky et al. (1984), DNA 3: 297-308). Interestingly, the IFN- α 1 (D) species has also a 100 times lower specific activity on human cells than other IFN- α species (T. Goren et al. (1983), Virology 130: 273-280), although it represents a large proportion of the total mass of leukocyte IFN (C. Weissmann (1981) in Interferon 3, Academic Press, N.Y. pp. 101-134). It is not excluded that some of the multiple IFN functions will be expressed more efficiently by IFN- β_2 than the antiviral effect. Despite their low concentration, growth-regulatory IFN- β induce HLA and (2'-5') oligo A synthetase more strongly than expected, have more prolonged effects and markedly inhibit the growth of the cells which produce them. The induction of IFN- β_2 by IL-1 and TNF suggests that it may play a role as an autocrine mediator of some effects of these cytokines in inflammation and acute-phase responses, as well as regulating cell proliferation.

Induction of mRNA which hybridizes with IFN- β_2 cDNA probes but not to IFN- β_1 cDNA has been observed in human cell lines after exposure to Interleukin-1 (IL-1) (J. Content et al. (1985), Eur.J.Biochem. 152: 253-257) and Tumor Necrosis Factor (TNF- α). The IFN- β_2 -specific immunocompetition assay with R-antibodies was used to examine if human fibroblasts FS11 cells indeed secrete the IFN- β_2 protein in response to these two cytokines (Figure 9). Both rIL-1 α and TNF- α induced the synthesis and secretion of the IFN- β_2 protein, to levels estimated by the immunoassay to be 10-20 U/ml IFN- β_2 . Optimal IFN- β_2 induction was seen with concentrations of rIL-1 α of 4 U/ml (0.13 ng/ml), similar to those required for IL-1 induction of prostaglandin E $_2$, collagenase and hyaluronic acid in human fibroblasts (J.H.Korn (1985), Arthritis Rheum. 28: 315-322). The optimal concentration of TNF- α for IFN- β_2 induction (400 ng/ml; 40 ng/ml) was higher than that required for the cytolytic effect on sensitive cells (0.1 ng/ml; A.M. Wang et al. - (1985) Science 228: 149-154) and even higher than that giving optimal growth stimulation of diploid fibroblasts (2 ng/ml). The onset of INF- β_2 secretion was somewhat faster with TNF- α reaching half maximal levels at about 6 hours, versus 8-12 hours with IL-1.

To the extent that it may be deemed necessary in order to complete the disclosure in this case, all of the subject matter of each of the publications referred to and cited hereinabove are hereby incorporated herein by reference.

It will be obvious to those skilled in the art that various other changes and modifications may be made without departing from the scope of the invention and the invention is not to be considered limited to what is shown in the drawings and described in the specification.

5

TABLE 1

10

Antiviral Activity of rIFN- β_2 * Produced in Transfected
Mammalian Cells

15

I. IFA-2 GENE

A. Constitutive expression in CHO cells

20

pSVIFA2-I	S1-15 cells	:	10-50 U/ml**
(control	CHO cells	:	0)

25

B. Transient expression in Cos7 cells

pSVIFA2-II	DNA	:	15 U/ml
(control IFN- β_1	pSVEIF DNA	:	500 U/ml)

30

II. IFA-11 GENE

A. Induced expression*** in CHO cells

35

Clone 1C40	:	400-500 U/ml
(control CHO cells	:	100 U/ml)

B. Induced expression in L cells

40

Clone LI-39	:	250 U/ml
(control L-TK ⁺ cells	:	< 4 U/ml)

45

* Assay with VSV in FS11 and WISH cells.
Neutralized by anti-IFN- β_1 .

50

** 3000 U/ml after concentration.

*** Induction with poly IC and cycloheximide for 10 hrs.
Harvest at 28 hrs.

55

TABLE 2

5 Antiviral Activity of Human rIFN- β_{2A} Produced in Hamster Cells
 Transfected and Amplified with IFN- β_{2A} cDNA

10

CHO-SVCIFB2 B132-10M	CLONE:	Human IFN activity* U/ml
B-131	(0 MTX)**	64
B-131-5M	(250 nM MTX)**	300
B-131-10M	(500 nM MTX)**	800

20

25 * Medium from cultures was collected every 24 hours and
 assayed with VSV in FS11 human cells. The activity
 was neutralized by anti-IFN- β_1 , but not anti-IFN- α or
 anti-IFN- γ .

30 ** Cultures were selected for resistance to indicated
 concentrations of methotrexate.

Claims

1. A recombinant human interferon-beta $_{2A}$ in purified form.
2. A recombinant human interferon-beta $_{2B}$ in purified form.
3. A cDNA consisting essentially of cDNA which codes for human interferon-beta $_{2A}$.
4. A cDNA consisting essentially of cDNA which codes for human interferon-beta $_{2B}$.
5. A cDNA having a nucleotide sequence substantially as shown in Figure 1.
6. A genomic clone capable of hybridizing to cDNA which codes for a human interferon-beta $_{2A}$ and is capable of expressing human interferon beta $_{2A}$.
7. A genomic clone capable of hybridizing to cDNA which codes for human interferon-beta $_{2B}$ and is capable of expressing human interferon-beta $_{2B}$.
8. A genomic clone according to claim 6, wherein the IFN-2 gene is contained.
9. A genomic clone according to claim 7, wherein the IFA-11 gene is contained.
10. A recombinant vector comprising a DNA sequence which codes for a human interferon-beta $_{2A}$ and further comprises regulatory regions which are positioned in a way that expression of the human interferon-beta $_{2A}$ is possible.
11. A recombinant vector according to claim 10, wherein said DNA sequence is a genomic DNA sequence.
12. A recombinant vector according to claim 10, wherein said DNA sequence is a cDNA sequence.
13. A recombinant vector according to claim 12, wherein the cDNA sequence is the sequence as depicted in Figure 1.
14. A recombinant vector comprising a DNA sequence which codes for a human interferon-beta $_{2B}$ and further comprises regulatory regions which are positioned in a way that expression of the human interferon-beta $_{2B}$ is possible.
15. A recombinant vector according to claim 14, wherein said DNA sequence is a genomic DNA sequence.
16. A recombinant vector according to claim 14, wherein said DNA sequence is a cDNA sequence.

17. A recombinant vector according to one of the claims 10 to 16, wherein the regulatory DNA sequences contain a promoter, which preferably is an SV40 early promoter.

18. A recombinant vector according to one of the claims 10 to 13, wherein the vector is pSVIFA2-I.

19. A recombinant vector according to one of the claims 10 to 13, wherein the vector is pSVIFA2-II.

5 20. A recombinant vector according to claim 13, wherein the vector is pSVCIF β_2 .

21. A cell line transformed by a recombinant vector according to one of the claims 10 to 20.

22. A cell line according to claim 21, wherein said cell line is eukaryotic.

23. A cell line according to claim 22, wherein said cell line is a hamster cell line.

24. A cell line according to claim 22, wherein said cell line is a CHO cell line.

10 25. A cell line according to claim 22, wherein said cell line is an L-TK cell line.

26. A cell line according to one of the claims 21 to 25, which is capable of producing human interferon-beta_{2A} and/or human interferon-beta_{2B}.

27. A cell line according to claim 24, wherein said cell line is CHO-SVCIF β_2 B132-10M.

28. A process for producing the human interferon-beta_{2A} and/or human interferon-beta_{2B} according to
15 claims 1 and/or 2, wherein a cell line according to one of the claims 21 to 27 is used.

29. The use of the human interferon beta_{2A} and/or human interferon-beta_{2B} as a pharmaceutical useful in the fields of inflammation, acute-phase response or regulation of cell proliferation.

20

25

30

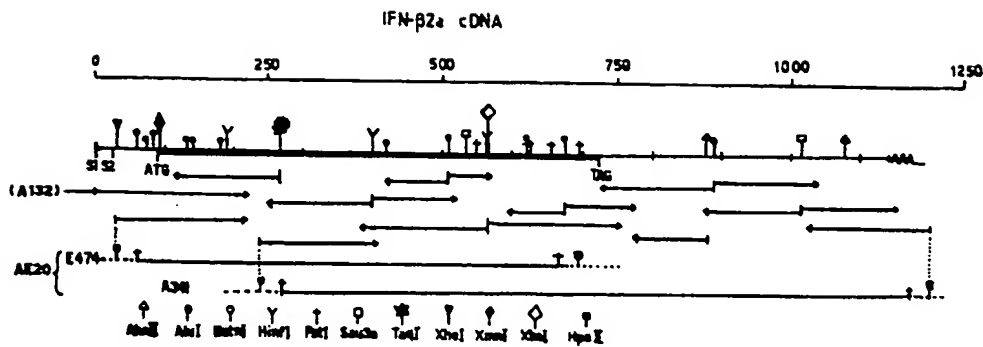
35

40

45

50

55



IFN-Beta 2a cDNA

```

10      20      30      40      50      60
AG GAC TGG AGA TGT CTG AGG CTC ATT CTG CCC TCG AGC CCA CCG GGA ACG AAA GAG AAG CTC
70      80      90      100     110     120
TAT CTC CCC TCC AGG AGC CCA GCT ATG AAC TCC TTC TCC ACA AGC GCC TTC GGT CCA GTT
Met Asn Ser Phe Ser Thr Ser Ala Phe Gly Pro Val
130     140     150     160     170     180
GCC TTC TCC CTG GGG CTG CTC CTG GTG TTG CCT GCT GCC TTC CCT GCC CCA GTA GCC CCA
Ala Phe Ser Leu Gly Leu Leu Leu Val Leu Pro Ala Ala Phe Pro Ala Pro Val Pro Pro
190     200     210     220     230     240
GGA GAA GAT TCC AAA GAT GTA GCC GCC CCA CAC AGA CAG CCA CTC ACC TCT TCA GAA CGA
Gly Glu Asp Ser Lys Asp Val Ala Ala Pro His Arg Gln Pro Leu Thr Ser Ser Glu Arg
250     260     270     280     290     300
ATT GAC AAA CAA ATT CGG TAC ATC CTC GAC GGC ATC TCA GCC CTG AGA AAG GAG ACA TGT
Ile Asp Lys Gln Ile Arg Tyr Ile Leu Asp Gly Ile Ser Ala Leu Arg Lys Glu Thr Cys
310     320     330     340     350     360
AAC AAG AGT AAC ATG TGT GAA AGC AGC AAA GAG GCA CTG GCA GAA AAC AAC CTG AAC CTT
Asn Lys Ser Asn Met Cys Glu Ser Ser Lys Glu Ala Leu Ala Glu Asn Asn Leu Asn Leu
370     380     390     400     410     420
CCA AAG ATG GCT GAA AAA GAT GGA TGC TTC CAA TCT GGA TTC AAT GAG GAG ACT TGC CTG
Pro Lys Met Ala Glu Lys Asp Gly Cys Phe Gln Ser Gly Phe Asn Glu Glu Thr Cys Leu
430     440     450     460     470     480
GTG AAA ATC ATC ACT GGT CTT TTG GAG TTT GAG GTA TAC CTA GAG TAC CTG CAG AAC AGA
Val Lys Ile Ile Thr Gly Leu Leu Glu Phe Glu Val Tyr Leu Glu Tyr Leu Gln Asn Arg
490     500     510     520     530     540
TTT GAG AGT AGT GAG GAA CAA GCC AGA GCT GTC CAG ATG AGT ACA AAA GTC CTG ATC CAG
Phe Glu Ser Ser Glu Glu Gln Ala Arg Ala Val Gln Met Ser Thr Lys Val Leu Ile Gln
550     560     570     580     590     600
TTC CTG CAG AAA AAG GCA AAG AAT CTA GAT GCA ATA ACC ACC CCT GAC CCA ACC ACA AAT
Phe Leu Gln Lys Lys Ala Lys Asn Leu Asp Ala Ile Thr Thr Pro Asp Pro Thr Thr Asn
610     620     630     640     650     660
GCC AGC CTG CTG ACG AAG CTG CAG GCA CAG AAC CAG TGG CTG CAG GAC ATG ACA ACT CAT
Ala Ser Leu Leu Thr Lys Leu Gln Ala Gln Asn Gln Trp Leu Gln Asp Met Thr Thr His
670     680     690     700     710     720
CTC ATT CTG CCG AGC TTT AAG GAG TTC CTG CAG TCC AGC CTG AAG GCT CTT CCG CAA ATG
Leu Ile Leu Arg Ser Phe Lys Glu Phe Leu Gln Ser Ser Leu Arg Ala Leu Arg Gln Met
730     740     750     760     770     780
TAG CAT GGG CAC CTC AGA TTG TTG TTG TTA ATG GGC ATT CCT TCT TCT GGT CAG AAA CCT
790     800     810     820     830     840
GTC CAC TGG GCA CAG AAC TTA TGT TGT TCT CTA TGG AGA ACT AAA AGT ATG AGC GTT AGG
850     860     870     880     890     900
ACA CTA TTT TAA TTA TTT TTA ATT TAT TAA TAT TTA AAT ATG TGA AGC TGA GTT AAT TTA
910     920     930     940     950     960
TGT AAG TCA TAT TTT ATA TTT TTA AGA AGT ACC ACT TGA AAC ATT TTA TGT ATT AGT TTT
970     980     990     1000    1010    1020
GAA ATA ATA ATG GAA AGT GGC TAT GCA GTT TGA ATA TCC TTT GTT TCA GAG CCA GAT CAT
1030    1040    1050    1060    1070    1080
TTC TTG GAA AGT GTA GGC TTA CCT CAA ATA AAT GGC TAA CTT TAT ACA TAT TTT TAA AGA
1090    1100    1110    1120    1130    1140
AAT ATT TAT ATT GTA TTT ATA TAA TGT ATA AAT GGT TTT TAT ACC AAT AAA TGG CAT TTAAAA...

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FIG.1

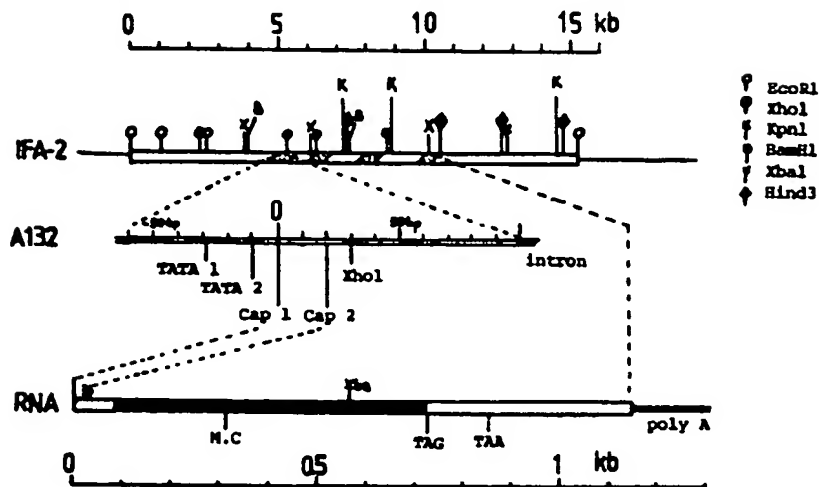
STRUCTURE OF HUMAN IFN β 2 GENE IFA-2

FIG.2

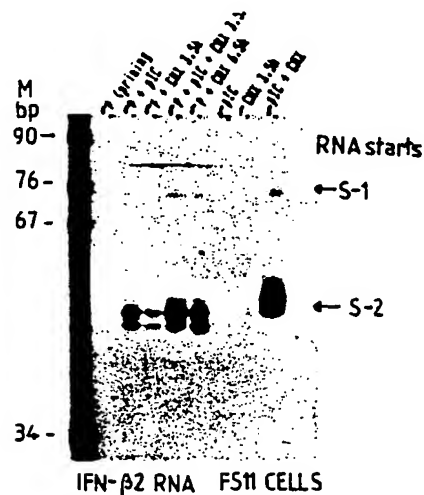


FIG.3

-74 -51
TAAATATAGGACTGGAGATGTCTGAGGCTCATTCTGCCCTCAG.....GatnI
S-1 xxa S-2

IFN- β 2 Gene, Clone IFA-2

IFA-2 -150 -140 -130 -120 -110 -100
ACTAAAGGACGTCACATTGCACAATCTTAATAAGGTTTCCAATCAGCCCCACCCGCTCTG

IFA-2 -90 -80 -70 -60 -50 -40
GCCCCACCCCTCACCCTCCAACAAAGATTATCAAAATGTGGATTTCCTCATGAGTCTCAA

IFA-2 -30 -20 -10 1 10 20
TATTAGAGTCTCAACCCCAATAAATATAGGACTGGAGATGTCTGAGGCTCATTCTGCC
cDNA ***** ATTCTGCC

TATA-1 TATA-2 S-1 S-2

IFA-2 30xha1 40 50 60 70 80
cDNA TCGAGCCACCGGGA-CGAAAGAGAAGCTCGATCTCCCTCCAGGAGCCAGCTATGAAC
TCGAGCCACCGGGAACGAAAGAGAAGCTCTATCTCCCTCCAGGAGCCAGCTATGAAC

IFA-2 90 100 intr1 110 120 130 140
cDNA TCCTTCTCCACAA GTAAGTCAGGAAATCCTTAGCCCTGAAGTCCAGCGGGTCGAGCC
TCCTTCTCCACAA GCBCCTTCGCTCCAGTTGCCTTCTCCCTGGGGCTGCTC....

IFA-2 150 160 170 180 190 200
CTGTGTGAGGGAGGGGTGTGTGCCCAAGGAGGGCTGGCGGGCGGCCAGCAGCAGAGGCA

IFA-2 210 220 230 240 250
GGCTCCAGCTGTGCTGTCAAGCTCACCCTGCCTCGCTCCCTCOC....

FIG.4

CONSTITUTE EXPRESSION OF HuIFN β 2 IN CHO CELLS

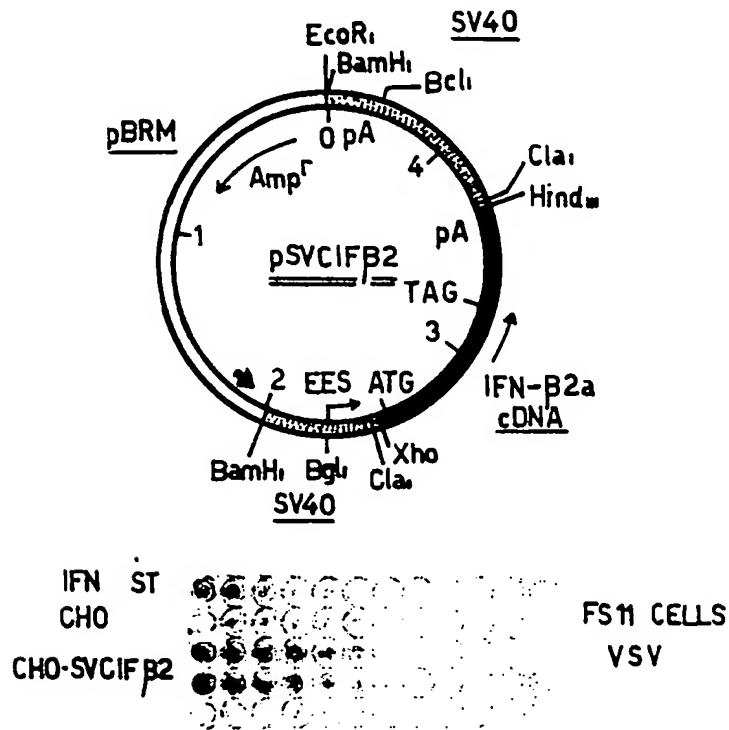


FIG.5

clone B131 250nMTX = 300 UNITS/ML (VSV, Mengo)

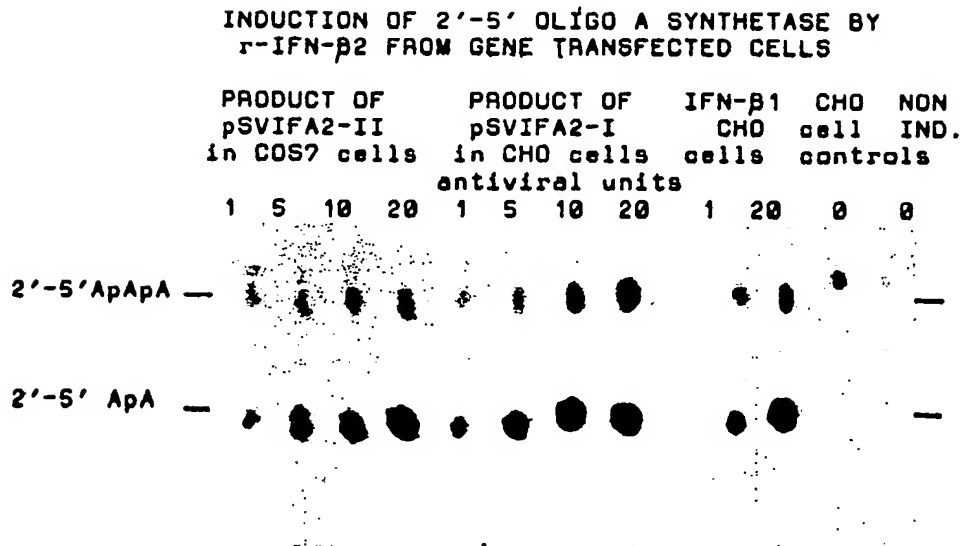


FIG.6

CONSERVED AMINOACIDS IN HUMAN TYPE I IFN α COMPARED TO IFN- β 2 SEQUENCE

IFN β -2 MNSF6

IFN β -2 TSAFGPVAFSLGLLLVLPAAFPAPVPPGEDSKDVAAPHRQPLTSSERTOKQIRYILOGIS^{oo o o 1 o}
 IFN β -1 -21 MTKCLLQIALLLCFSTTALS
 IFN α -A -23 MALT FALL VALVVL SCKSSCS
 IFN α -C MALSFS LI MAVI VLSYKICS
 IFN α -II MVLLI PLLVALPLCHCGPVGS

IFN β -2 ALRKETCNKSNWCESSKEALAENNLNLPKMAEKD-GCFQSGFNEETCLVKIITGL^{o o o o o}
 IFN β -1 MSYNLLGLQAGSNFQCQKI L WQI NGRLEYCLNDRMNFDIPE-FIKQLQQFQKED^{o o o o o}
 IFN α -A VGC DL PQTHSLOSARTL MLLAQMRKISLFSCLNDRHDFGFPQ-EEF-GNQFQKAE
 IFN α -C LGCDLPQTHSLGNRRAL ILLGQMGRI SPFSCLNDRHDFRIPQ-EEFDGNQFQKAE
 IFN α -II LSCDLPQNHGLLSANTLVLLHQMARRISPFLCLNDRHDFRFPQ-FMVKGSQLQKAM^{N SE D M S S M E QP DK P}

IFN β -2 LEFEVYLEYLQNR^{o o o o}ESSEEQARAVQMSTKVLIQFLQKKAKNLDAI-----
 IFN β -1 AALTTY-EMLNIFAI FRQSSSTGWN ETIVENLLANVYH-QINHLKTVLEEKLEK
 IFN α -A TIPVLH-EMIQQIFNLFSTKSSAAMDETLLDKFYTELYQ-QLNDLEACVIGVGV
 IFN α -C AISVLH-EMIQQT FNLFSTEDSSAAMQSLLENFSTELYQ-QLNDLEACVIGVGV
 IFN α -II VMSVLH-EMLQQIFSLFHTERSSAAMWMTLLDQLHTELHQ-QLQHLETCLLQVVGE^{IM T N TL D CI D M VLCD FA}

IFN β -2 -TTDPTTNASLLT-----KLQAGNQWLQDMTHLILASFKEF--LQSSLRALRQM#^{oo o o o o o o o o}
 IFN β -1 EDFTRGKLMSSHLKRYYGRI LHYLRAKEYSHCAWTIVAV--EILAHFYFINAL TGYLAN#
 IFN α -A TETPLMKEDSILAVRKYFQRITLYLKEKKYSPCAWEVVRA--EIMRSFSLSTNLQESLRSKE#
 IFN α -C EETPLMNE DSILAVRKYFQRITLYLIERKYSPCAWEVVRA--EIMRSLSFSTNI QKRI ARKD#
 IFN α -II GFSAGAISSPALT LRAYFQGI RYVLKEKKYSCAWEVVRA--EIMKSLFLSTNMQERLASKDRDLGSS#^{I S YV K R T S IIF}

FIG.7

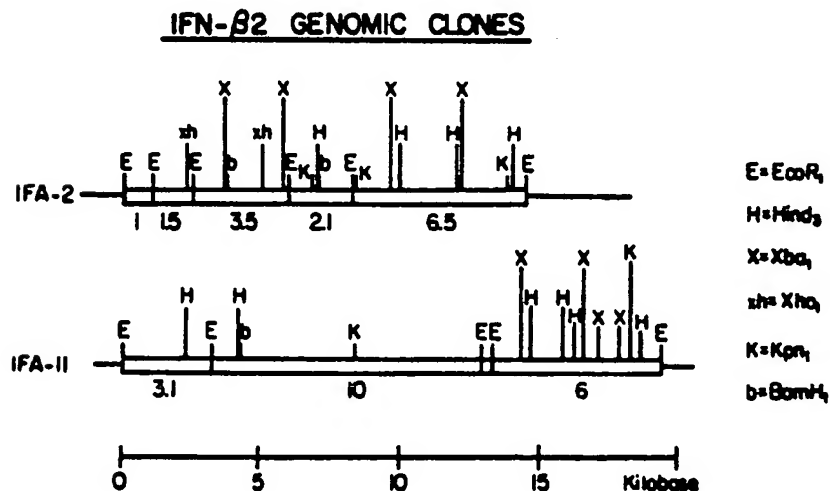


FIG.8

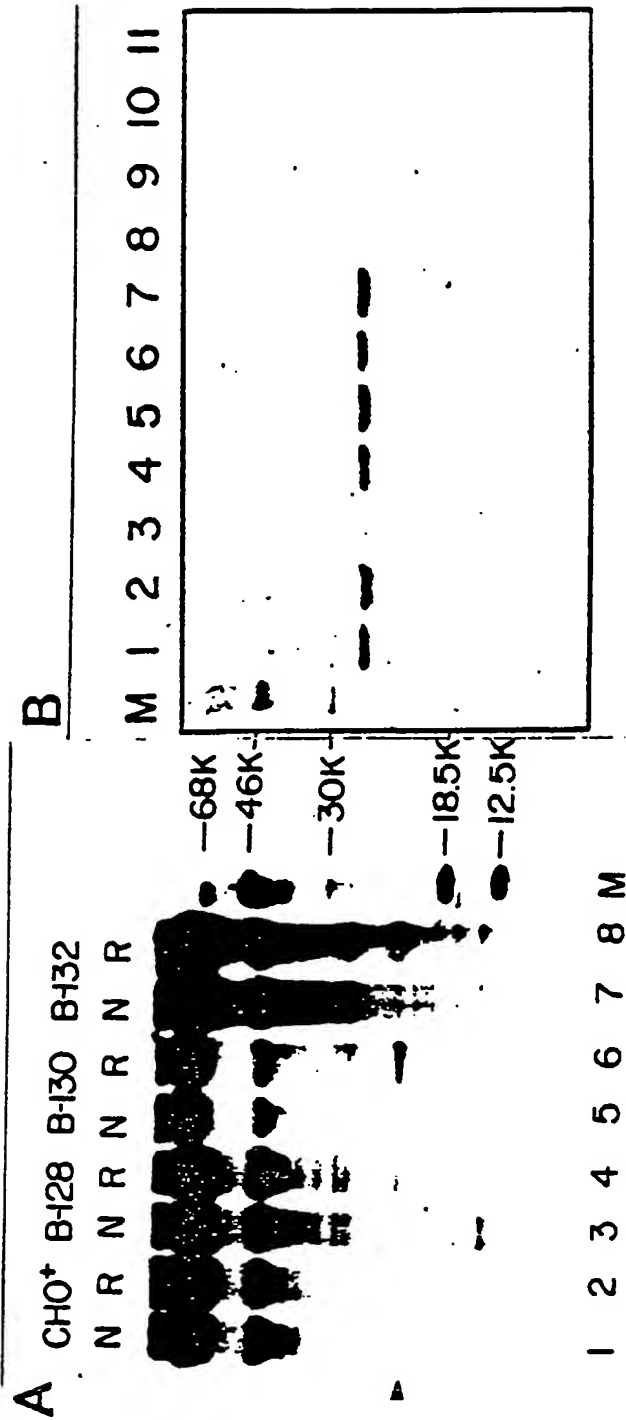


FIGURE 9



European Patent
Office

EUROPEAN SEARCH REPORT

Application number

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 86114049.9
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
D,A	DE - A1 - 3 043 981 (YEDA) * Claims * --	1-7, 21,22	C 12 N 15/00 C 12 P 21/00 C 07 K 15/00 A 61 K 45/02
D,A	EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 120, no. 1, November 2, 1981, Springer-Verlag Berlin, Heidelberg, New York MORY et al. "Synthesis of human Interferon β_1 in Escherichia coli Infected by a Lambda Phage Re- combinant Containing a Human Genomic Fragment" Pages 197-202 * Totality * --		
D,A	NUCLEIC ACIDS RESEARCH, vol. 12, no. 18, September 25, 1984, London U. NIR et al. "A human INF- β_1 gene deleted of promotor sequences up- stream from the TATA box is con- trolled post - transcriptionally by dsRNA" Pages 6979-6993 * Totality * --		TECHNICAL FIELDS SEARCHED (Int. Cl. 4) C 12 N C 12 P C 07 K A 61 K
A	JOURNAL OF INTERFERON RESEARCH, vol. 4, no. 1, 1984. New York P.B. SEHGAL et al. "Detection of Human Beta - 2 Interferon Using a Radioimmunoassay" Pages 63-66 * Totality * ----		
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 19-01-1987	Examiner BECKER
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

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